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Enhancing Bacterial Expression of Mammalian GProtein Coupled Receptors The Creation of a Proteorhodopsin-Bovine Rhodopsin Chimera

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Enhancing Bacterial Expression of Mammalian G-Protein Coupled Receptors

The Creation of a Proteorhodopsin-Bovine Rhodopsin Chimera

A Capstone Project Submitted in Partial Fulfillment of the Requirements of the Renée Crown University Honors Program at Syracuse University

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Honors Capstone Project in Biochemistry

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ABSTRACT

Bovine (cow) rhodopsin is a 7-transmembrane (7TM), light-absorbing protein located in rod cells. It is activated by the photoisomerization of retinal, a Vitamin A derivative. This light-triggered reaction activates the G-Protein Coupled Receptor (GPCR), resulting in a signaling cascade within the cell. When previously cloned in an *E. coli* expression vector in the Braiman Lab, bovine rhodopsin expression was not successful, possibly due to *E. coli*'s lack of recognition of the foreign N-terminus portion of the protein, which may be a prerequisite for proper folding and insertion into the membrane. Our proposed solution is to create a chimera protein, replacing the N-terminal leader sequence of bovine rhodopsin with proteorhodopsin's N-terminal leader sequence in order to successfully express this chimera in E. coli using the pBAD-Topo® plasmid. Proteorhodopsin (pR), a protein found in marine bacterioplankton, with analogous photochemical activity to bovine rhodopsin, was chosen to serve as a candidate for creating this chimera due to its robust, successful protein expression, giving around 10mg/L of culture, expressed in the same plasmid and bacterial system. Using the restriction enzyme NcoI, we removed a portion of the gene encoding the N-terminus of bovine rhodopsin, while PCR followed by NcoI digestion was used to amplify a similar-sized portion of pR's N-terminus encoding DNA. These two DNA fragments were ligated, using T4 Ligase. Confirmation of successful ligation of these two DNA fragments to create the chimera protein-encoding DNA is still pending.

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ACKNOWLEDGEMENTS

First and foremost, absolutely none of this could have been done without the constant caring and intelligent instruction under Professor Braiman. I thank him so much for taking me into his lab and allowing me to perform research under his supervision. I would also like to thank Farhana Syed for being there every step of the way, guiding me one-on-one in such a sincere fashion, and Professor Spencer for his generosity to be my Capstone reader. Other members of the Braiman lab as a whole are absolutely terrific, and working with them has made the experience that much more enjoyable, so I say thank you.

The Honors Program has been extremely wonderful in their advising and support, and they broadened my perspective through the honors program. Last but not least, I would also like to thank my dearest family, friends, and mentors who have shown constant moral support through my time here at Syracuse University.

Advice For Future Honor Students

My number one piece of advice for honor students, which is applicable for everyone, is *do what you love and give it your all*. As simple as this advice may seem, you will get so much more from any experience when you do this, and it'll be beyond rewarding.

CHAPTER 1

GPCRs and Rhodopsin: And Introduction

WHAT ARE GPCRs?

GPCRs, or G Protein-coupled receptors, make up the largest superfamily of proteins in the body (Gether, 2000). They all have many similar structures in common, such as an extracellular N terminus with a cytoplasmic C terminus (Ballesteros 1994).

GPCRs are seven-transmembrane (7TM) proteins. Each GPCR consists predominantly of alpha-helical domains, which span the cellular membrane and are connected by interhelical loops. The helices collectively span the cellular membrane seven times, hence the name "7TM."

GPCRs' MECHANISM OF ACTION

The ability of GPCRs to employ and control intracellular heterotrimeric G proteins is the source of its name (Gether, 2000). When an extracellular ligand binds and activates the GPCR, an intracellular cascade of events is triggered through conformational changes, starting with the α, β, γ subunits of the aforementioned heterotrimeric G proteins (Gether, 2000). These three subunits can be regarded as two separate functional units, with the α subunit acting as its own functional unit, and the β and γ subunits considered the second, as they have a close, tight interaction (Cabrera-Vera et al., 2003).

When the initial GPCR conformational changes occur, the binding of GTP follows GDP's release from the α subunit (Bourne et al., 1991). The newly GTP-bound α subunit – which leaves the stable βγ-dimer – and the βγ-dimer itself both have the potential to stimulate or inhibit signaling pathways intracellularly; including but not limited to: adenylate cyclases, phospholipase activation, as well as the activity of the potassium and calcium channels. The collection of these complex-signaling pathways affects the cells in multiple ways, usually resulting in a physiological response (Hamm, 1998).

GPCR IMPORTANCE

G-Protein Coupled Receptors are important in the pharmaceutical industry, as many pharmaceuticals act upon G protein-coupled receptors as either agonists or antagonists. Pharmaceutical scientists focus on these receptors as a target due to the prevalent role of GPCRs in human physiology. At least 60% of target molecules for existing drugs reside on the cell exterior, and GPCRs make up half of them (Overington et all, 2006). Additionally, dysfunctions in these GPCRs cause many human diseases, demonstrating the importance for their research (Kristiansen, 2004).

Many of the receptors that these GPCRs comprise include: hormones, paracrines, neurotransmitters, and neuromodulators. The diversity of ligands that activate these GPCRs include: amines, peptides, amino acids, glycoproteins, fatty acids, phospholipids, glycoproteins, and

calcium ions. Sensory receptors, including rhodopsins, are activated by ligands including: odorants, bitter and sweet tastants, pheromones, and photons (light). Knowing what kind of GPCR a protein is, as well as what type of ligands activate it are important, because this information is useful in creating drugs for such GPCRs (Kristiansen, 2004).

GPCR MUTATION BENEFIT: THE CCR5 GENE & HIV INHIBITION

An example of the great significance of GPCR mutations is their involvement with HIV's entry mechanism into human immune cells. HIV uses the chemokine (C-C) motif receptor 5, commonly known as CCR5, for entry and proliferation; and mutation in the CCR5 gene result in a change of its GPCR structure that blocks HIV's cell entry. This terminates proliferation, and essentially stops the progress of the retrovirus from taking control of its host (Unutmaz et al., 1998). This is a rather interesting example of a GPCR mutation, as the mutation seems to benefit us rather than cause disease, and it is for this reason that it is worth noting. Maraviroc, the first of a series of drugs that have been designed to bind to the CCR5 GPCR and mimic this beneficial mutation, thereby blocking HIV entry into cells, received FDA approval for treatment of AIDS in 2007 ("Maraviroc").

PROTEIN PURIFICATION

In order to get a pure protein crystal, the protein must first be purified. There are many methods for protein purification, but one very

relevant protein purification technique that is currently undergoing optimization by the Braiman Lab, is selective citrate-induced precipitation (R. Partha and M. Braiman, U.S. Patent Pending).

The Braiman lab has shown that a particular 7-helix protein, proteorhodopsin (pR), has, what appears to be, at least one binding site for citrate. They concluded this by observing pR's aggregation and precipitation by use of citrate. The Braiman lab is currently finding optimal conditions for purifying these GPCRs, which may be useful for purification for the protein chimera we are attempting to create, especially since the chimera is planned to incorporate part of pR.

GPCR OF INTEREST: BOVINE RHODOPSIN

Bovine Rhodopsin, which is isolated from cows, is a mammalian7-transmembrane (7TM) light-absorbing protein located in the eye, particularly the rod cells ("Rods and Cones"). It is activated by the photoisomerization of retinal, a Vitamin A derivative, which results in a conformational change from 11-*cis*-retinal to all-*trans*-retinal, trigger by light, which is shown below.

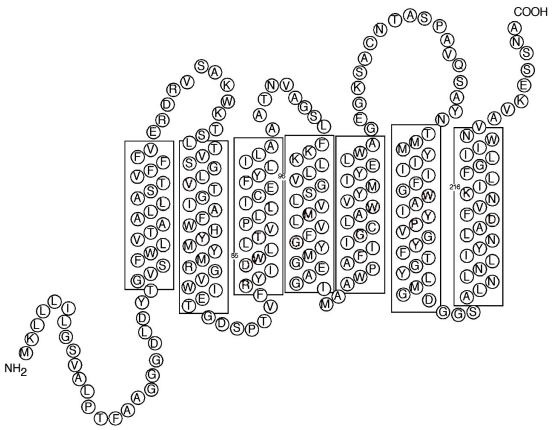
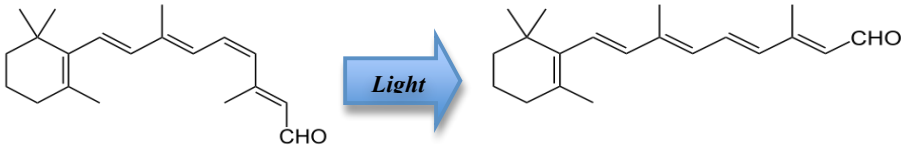


Figure 1 a 2D amino acid protein structure of Proteorhodopsin (Béjà, et al., 2000).



This light-triggered reaction activates the G-Protein Coupled Receptors signaling activity, resulting in a cascade affect within the cell due to conformational changes, which, as mentioned before, is typical for all GPCRs.

When previously cloned in an *E. coli* expression vector in the Braiman Lab, bovine rhodopsin protein expression was apparently unsuccessful. That is, when 11-cis retinal and arabinose were added, the bacteria did not develop any red color, which suggests that the protein was not present in the cellular membrane. However, there is a possibility that the protein was expressed, but due the large size or foreignness of its N- terminus head, it might not have been possible to re-fold properly into the cellular membrane.

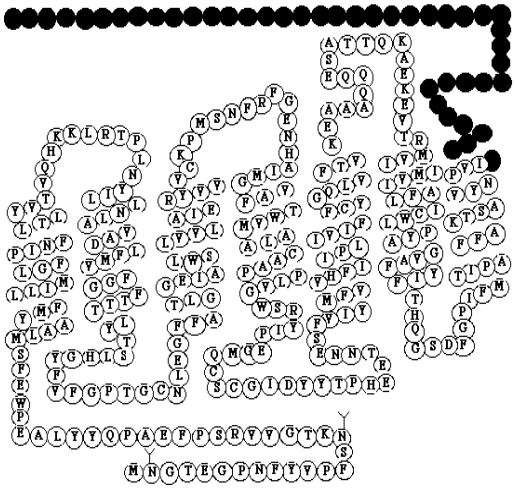


Figure 2 a 2D amino acid protein structure of bovine rhodopsin (Yeagle et al., 1997).

We hypothesize that the issue mentioned above may be due to improper folding, as well as *E. coli*'s lack of recognition of the foreign N-terminus head portion of the protein. We believe this may be the problem because N-terminal leader and signal sequences have been shown, especially in the case with integral proteins, to control protein expression.

Literature showed an example where this was the case. Bacterio-opsin expression was not robust, and several N-terminal amino acids were changed to increase the A-T content (Karnik, 1987). The result was a decrease in *E. coli* expression, or cellular membrane insertion, thus, showing that the N-terminus is critical in determining the ability for proteins to be expressed cellularly (Karnik, 1987).

SOLUTION: PROTEIN CHIMERA

Our solution is to create a chimera protein, a protein hybrid. Protein chimeras have been used before to fix such problems, and therefore, we believe it may serve as a solution to better fully express bovine rhodopsin.

Since we hypothesize that the problem may lie with the N-terminus, we plan on fixing this solution by replacing the N-terminal leader of bovine rhodopsin with proteorhodopsin's N-terminal leader sequence, in the hope that it would allow for successful protein expression and folding in *E. coli* by using the pBAD-Topo® plasmid. We chose to use proteorhodopsin in the creation of this chimera for several reasons.

GPCR CHIMERA CANDIDATE: PROTEORHODOPSIN

Proteorhodopsin (pR), discovered in 2000, is a purple-colored 7TM protein found in marine bacterioplankton, with analogous photochemical activity to bovine rhodopsin (Béjà, et al., 2000).

Expression of pR in *E. coli* is robust, producing around 10mg/L. Due to pR's successful expression, using the N-terminal leader sequence of pR and replacing bovine rhodopsin's N-terminal leader sequence, we could ideally create a chimera expression of bovine rhodopsin to further study this GPCR.

GENE CLONING & PROTEIN EXPRESSION

The central dogma in molecular biology, which many know it as, is used by biochemistry among other scientists for cloning. The central dogma is simple: DNA → RNA → Protein. Therefore, while we are concerned with the protein, rhodopsin, and making the chimera, we work with the DNA that encodes, or makes, the protein, which will later be expressed as a protein by *E. Coli*.

Consequently, to make our chimera protein, we have to make a chimera plasmid DNA – the hybrid. To make this chimera plasmid, we simply cut and paste DNA by use of enzymes, called endonucleases, as well as T4 Ligase. The specifics of this process thoroughly explained in the following chapter.

CHAPTER 2

METHODOLOGY & EXPERIMENTATION

EXPERIMENT

First and foremost, media for bacterial growth had to be made. The ingredients of 1L of the enriched media, based on LB (Luria-Bertani) media were:

Ingredient	Quantity
Tryptone	10 g
Yeast Extract	6 g
Na_2HPO_4	6.8 g
Potassium Phosphate	7.56 g
Glycerin (glycerol)	10 mL

These ingredients were typically made up in a 1.5-liter quantity in a 3-l flask. Prior to autoclaving, the pH was brought to 6.73 with NaOH using a pH meter.

To obtain plasmid DNA to work with, cultures containing (separately) expression plasmids for either bovine rhodopsin or proteorhodopsin were grown. Initial attempts to isolate a high concentration of both DNA plasmids were unsuccessful. Therefore, a larger quantity was grown. Approximately 49mL of bacterial culture containing, each plasmid, bovine rhodopsin and pR, were grown. For each

plasmid, there were seven, 15-mL tubes with 7mL of culture.

The plasmids (bovine rhodopsin and proteorhodopsin) were isolated from bacterial-grown cultures using the Wizard®Plus SV Minipreps DNA Purification System ("Wizard Plus SV Minipreps DNA Purification System"). The Spin Column was transferred to a new, sterile 1.5mL centrifuge tube, leaving behind the Column Wash Solution.

1. The Spin Column for each tube was transferred to a new, sterile 1.4mL centrifuge tube.

2. The plasmid DNA was eluted by adding 70µl of Nuclease-Free Water to the Spin Column. It was centrifuged for 1 minute at maximum speed at room temperature. (*Note*: Typically 100µl of Nuclease-Free Water is added, but we added 70µl for a higher concentration of DNA for more efficient results later on.)

3. After this, the assembly from the 1.5mL centrifuge tube was removed and the Spin Column was discarded.

4. The DNA plasmid was stored (without any buffer) at -20°C.

A total of 70µl of plasmid DNA was isolated for bovine rhodopsin and pR DNA plasmids. A gel was run to confirm that both bovine rhodopsin and proteorhodopsin DNA plasmids were isolated, which can be seen in the results, Figure 4.

GEL ELECTROPHORESIS

Gel electrophoresis separates on the basis that DNA is negatively charged, and since we know that smaller, less supercoiled DNA travels faster, we can separate fragments of DNA by size and compare the fragments to already known bands that appear from using a ladder, which can be seen in the results, in Figure 4.

In order to do this, however, we had a to make a 0.8% agarose gel, which is place in the gel apparatus. The protocol followed was:

1) In 150mL flask 0.4g agarose and 50mL 1xTBE buffer (2mM EDTA, 90mM Tris base, 90mM boric acid) was added.
2) The mouth of the flask was loosely with plastic wrap, and swirled to dissolve the agarose.
3) The flask was heated in the microwave for 4 min, swirling to dissolve all of the agarose once the time was up.
4) The plastic wrap was removed, and a drop of ethidium bromide was added and swirled.
5) The liquid gel was then poured into the gel apparatus for solidification.
6) When the gel electrophoresis was run, it was run at 125V for 1-2 hours.

THE POLYMERASE CHAIN REACTION

The Polymerase Chain Reaction (PCR) is an extremely helpful technique exploited to at least amplify DNA. The general schema for the PCR is as follows (Kubista et al., 2006):

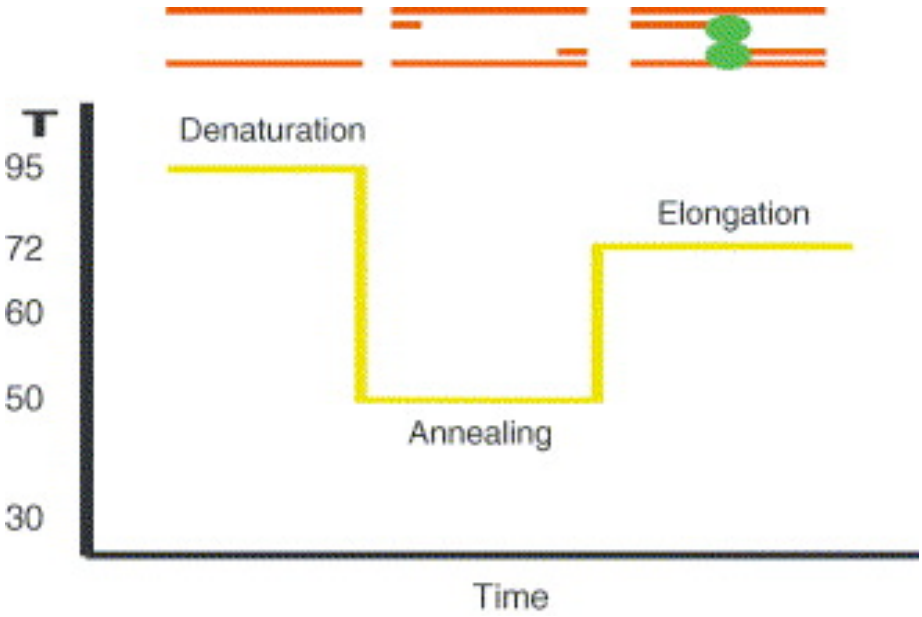
1) A DNA template is needed, which can be double or single stranded.

2) From this template, two oligonucleotides, called "primers," are created. These primers – one forward and the other reverse – are used to bind to specified regions on the DNA template. Thus, the nucleotides should be complementary, resulting in double hydrogen bonding between adenine and thymine, triple hydrogen bonding with guanine and cytosine. (*Note*: A base, or nucleotide, can purposely be changed in the primer, resulting in uncomplementary matching. This results in mutations, such as a "point mutation," which is the changing of only one nucleotide).

3) The building blocks for making copies called dNTPs, or deoxy-nucleotide-tri phosphate. These are used for the elongation stage. (*Note*: ddNTPs, dideoxynucleotide-tri-phosphate, can also be used; however, these are added for terminating elongation, which gets randomly added. This is usually used in forensics for DNA matching.)

4) A heat-stabile polymerase is needed. This enzyme is required for the elongation phase, as well as a buffer. (*Note*: the dNTPs,

Polymerase enzyme, and buffer can usually be in a “mastermix” for convenience, which is used in this experiment.)



Above is a table that shows temperatures needed for a typical PCR run. At 95°C, denaturation, or separation of the two complimentary strands of DNA occur to allow the annealing, or binding, of the oligonucleotides (primers), which occurs when the temperature is the lowered to approximately 50°C (Kubista et al., 1996). Elongation then proceeds at 72°C as the polymerase binds, adding the complementary nucleotides to finish replication. The newly synthesized DNA strands then are used and further templates, with replication increasing at an exponential rate.

In this experiment, proteorhodopsin’s N-terminal DNA leader sequence was amplified by using the Polymerase Chain Reaction (PCR) method. The constituents and amount for the PCR reaction mixture used

were:

Ingredient	Volume
TCM+ (Proteorhodopsin) DNA	5µl
Forward Primer 5'ATGCCATAGCATTTTTATCC 3'	1µl
Reverse Primer 5' AACACCCCATGGATCACTAGCATCAAGGTCACCACCACCTGC'3	1µl
AmpliTaq (Promega)	25µl
H_2O (Sterile)	18µl
	Total: 50µl

The PCR Program used to amplify proteorhodopsin N-terminal region was:

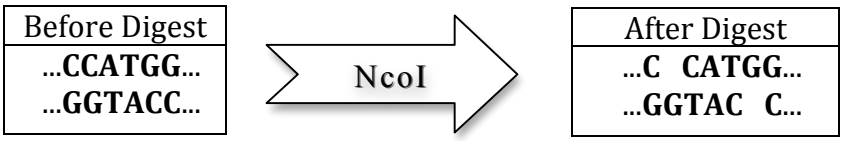
	Temperature	Time
1 Cycle	97°C	1min
	34°C	1min
	72°C	1min
35 Cycles	95°C	1min
	54°C	1min
	72°C	1min
1 Cycle	72°C	1min
	4°C	*Hold*

A Gel was run to confirm that the PCR worked, which can be seen in the results, Figure 4. The exact predicted length of the PCR product is 227 base pairs.

THE PLASMID RESTRICTION ENZYME DIGEST

A restriction enzyme, or endonuclease, is one that cleaves DNA based on short, specific, double-stranded DNA sequence for a specific site, known as a recognition site. This occurs in-between the ends of the DNA sequence, hence "endo-." (*Note*: exonucleases are enzymes that chew DNA from the ends). The recognition sites tend to be palindromic, although not always the case. Restriction digests can be prevented through methylation (Arber et al., 1969).

The restriction enzyme used in this lab is NcoI. The site that NcoI recognize and cuts, and the result of this reaction, are shown below (Yuan, 1981; Bickler 1993):



(*Note*: "…" represents a continuation of nucleotides)

In this project, the constituents for the PCR product digest with NcoI used was:

Ingredient	**Volume**
PCR Product	30µl
Buffer4 (Promega)	3.5µl

NcoI (Promega)	1.2µl
	Total: 34.7µl

The PCR Product with NcoI digest was incubated at 37°C for 1.5 hours. The constituents for the Bovine Rhodopsin digest with NcoI used was:

Ingredient	**Volume**
Bovine Rhodopsin DNA	15µl
Buffer4 (Promega)	1.5µl
H_20 (Sterilized)	5.5µl
NcoI (Promega)	3.0µl
	Total: 25µl

The bovine rhodopsin digest was incubated at 37°C for 1.5 hours.

A gel was run to confirm that the NcoI did in fact cut bovine rhodopsin and proteorhodopsin, which can be seen in the results, in Figure 4.

THE PHOSPHATASE ENZYME REACTION

The purpose of using the phosphatase enzyme is to prevent re-annealing of the plasmid to allow a better chimera yield. This does this by removing the phosphate group by hydrolysis; acting opposite of what a kinase does.

This reaction proceeded for 1 hour at 37°C. The ingredients added

to the NcoI digested Bovine Rhodopsin Plasmid were:

Ingredient	Volume
Alkaline phosphatase buffer (Promega)	5.5µl
Alkaline phosphatase (Promega)	1µl
H_20 (sterile)	2µl
	Total: 5µl

ETHANOL PRECIPITATION

Ethanol precipitation is used for getting rid of salts in nucleic acid solutions, and concentrating the nucleic acid desired, which is DNA in this case. Salt and ethanol are both added to the solution containing DNA, which precipitates DNA, later to be separated by centrifugation. After this protocol is followed, a pellet usually results, which is then re-dissolved in water. This was done for the NcoI digested bovine rhodopsin plasmid (Oswald). The protocol followed is below.

1) .1µl of glycogen stock solution was added to the NcoI digested bovine rhodopsin plasmid.

2) 7µl of 0.3M sodium acetate was then added.

3) 2 volumes of 100% ethanol (95% was used in this experiment) were used, which amounted to 150µl of ethanol added.

4) The Eppendorf tube containing this mixture was set on dry ice for

20 minutes.

5) The Eppendorf tube was then centrifuged at maximum speed for 20 minutes at room temperature.

6) After decanting the supernatant and being careful to leave behind the visible or invisible DNA pellet, 50µl of 70% ethanol was added to wash the DNA.

7) Following decanting of the supernatant, the Eppendorf tube blotted on KIMWIPES® and the Eppendorf tube was air-dried for 15 minutes.

8) The DNA was resuspended in 10µl of sterile water.

THE LIGATION REACTION

The purpose of the ligation reaction is to recombine, or, essentially "paste" the DNA together. The T4 Ligase enzyme forms the bond between the 3' hydroxyl and 5' phosphate ends, which results in a covalent phosphodiester bond. In this case, the "pasting" site is the NcoI site, where we used the enzyme to cut the DNA.

For the ligation reaction of pR PCR product and bovine rhodopsin digested plasmids with NcoI, 3 ligations were done. Two were run with different volumes of Vector (bovine rhodopsin digest plasmid) and Insert (pR PCR product), and the last was a control (without any DNA). The constituents of these 3 reactions were:

	Reaction1	*Reaction 2*	*Reaction3*
Ingredient	**Volume**	**Volume**	**Volume**
Vector	5.0µl	2.0µl	2.0µl
Insert	7.5µl	10.5µl	0µl
H20 (Sterile)	0µl	0µl	10.5µl
Buffer	1.5µl	1.5µl	1.5µl
T4 Ligase (Promega)	1.0µl	1.0µl	1.0µl
	Total: 15µl	Total: 15µl	Total: 15µl

These 3 reactions tubes were places in 15°C water bath for an hour.

TRANSFORMATION

Bacterial transformation is the transfer of bare DNA, or genetic material, which can also occur between strains of bacteria. The purpose of this is to allow bacterial integration of the DNA for later replication of the once bare DNA. Most importantly, bacterial also allows for protein expression to proceed following bacteria's integration of the DNA, which, in our case, serves as the purpose of making the desired protein chimera ("Bacterial Transformation").

After the ligation step, we attempted to transform the ligated DNA plasmid into competent cells for protein expression. To do this, the following was done:

1. 50µl of XL1-Blue Super Competent *E. Coli* cells were added to each of the three the Eppendorf tubes containing the ligation product.

2. The Eppendorf tubes were left on ice for 20 minutes, heat shocked at 42°C for 45 seconds, and set on ice for 1 minute.

3. Following this, 200µl of antibiotic-free LB medium was added, and left to recover at 37° for 1.5 hours.

4. Lastly, on 2 separate plates for each reaction tube, 100µl and 50µl of the transformants were separately plated on LB + Ampicillin plates, giving a total of 6 plates.

CHAPTER 3

RESULTS & DISCUSSION

The phosphatase reaction was an important alteration to the prior protocol followed to create the DNA plasmid chimera. This step is not always taken in making a chimera, but we decided that we needed to heighten our chances of yielding a product because previous attempts of creating a chimera failed. We thought that this could fix the problem by preventing the original vector (for bovine rhodopsin expression) from self-annealing.

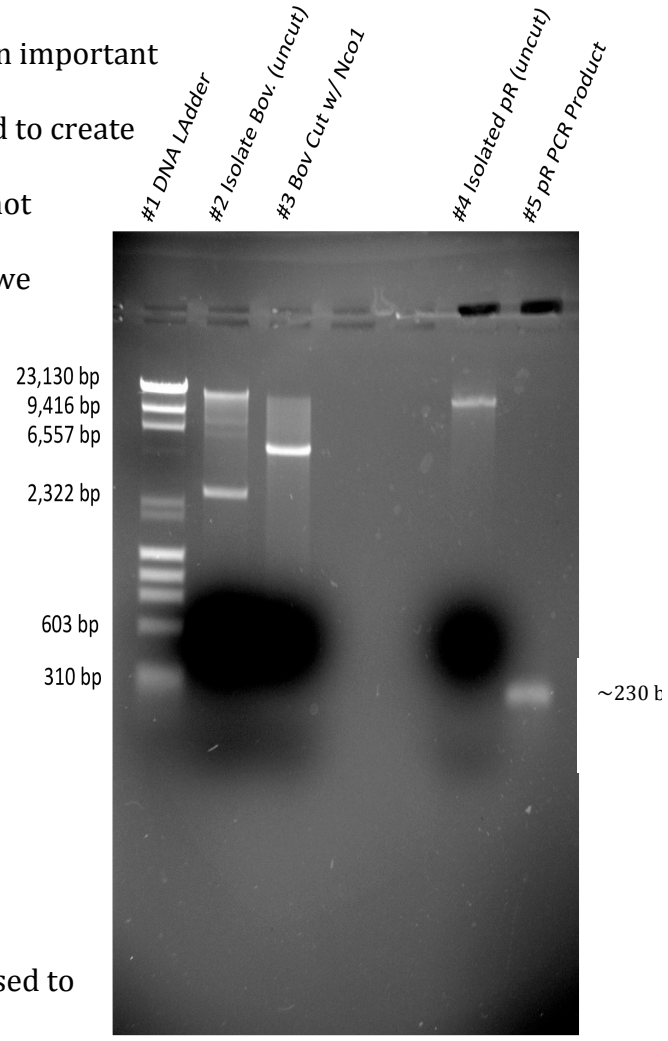


Figure 4 Results from gel electrophoresis.

During the course of these experiments, gel electrophoresis was used to confirm progress before continuing. As previously mentioned, gel electrophoresis separates on the basis that DNA is negatively charged, and since we know that smaller, less supercoiled DNA travels faster, we can separate fragments of DNA by size.

All of the results are conveniently collected in one gel

electrophoresis picture shown in Figure 4. In the first lane is the DNA ladder. The ladder consists pre-cut DNA bands with known sizes, which serve as a standard for determining the sizes of unknown DNA samples run in subsequent lanes.

In lane 2 is the isolated DNA plasmid, pBAD-TOPO+bovine rhodopsin. This plasmid is expected to have about 5.1kb (kilobasepairs), and the presence of the bands in lane 2 confirms that pBAD-TOPO+bovine rhodopsin was isolated.

The reason for the band just above 2,322bp is due to the circular plasmid supercoiling very compactly, allowing the plasmid to actually move faster than a linear DNA with the same number of base pairs. The band above 9,416bp is the supercoiling of the DNA which is supercoiled, and bulky DNA, resulting in a slowing traveling band.

Lane 3 shows the result of the restriction enzyme digest of bovine rhodopsin with NcoI, with a band around 5kb. This is expected as pBAD-TOPO plasmid is around 4.1kb, and the bovine rhodopsin gene that was previously cloned into *E. Coli* is 1050bp, which amounts to 5.15kb. Therefore, this NcoI digest of pBAD-TOPO+bovine rhodopsin was successful.

In lane 3, we also see some smear, however, there is only one prominent band as the plasmid is now linear due to the enzyme cutting the DNA. Therefore, only one state is favored in terms of supercoiling.

Lane 4 shows the isolated pBAD TOPO+proteorhodopsin plasmid DNA. There is not much of a smear here, with a prominent band between 9kb and 23kb. This is expected, for the same reason given for bovine rhodopsin. The band confirms that our DNA was isolated from bacterial cultures.

Lane 5 has a band at the expected length around 227bp. This confirms that the proteorhodopsin PCR did indeed work, which consequently was used in the attempt of creating the DNA plasmid chimera with the NcoI cut bovine rhodopsin.

Now while we have successfully completed the steps in creating the chimera, confirmation of success is still being conducted within the lab, and the experiment is being redone to confirm that the appropriate steps can be replicated and produce the same, if not better, results.

For those who continue this project, after the creation of the plasmid chimera has been made, characterization of protein expression must be done, and the optimization of its isolation with techniques, such as citrate purification and nickel column affinity must be conducted in order to attempt protein crystallization.

Additional Figures

Figure 5: Shows primers binding to pR's DNA for amplification of this sequence encoding pR's N-terminus leader sequence. The bold box base pairs show NcoI site. An NcoI site is also incorporated by in primer 2.

Figure 6: A 2D amino acid protein structure of Proteorhodopsin

Figure 7 – Sequence of expected PCR product, with 227bps. The bold and boxed based pairs correspond with the Nco1 restriction sites.

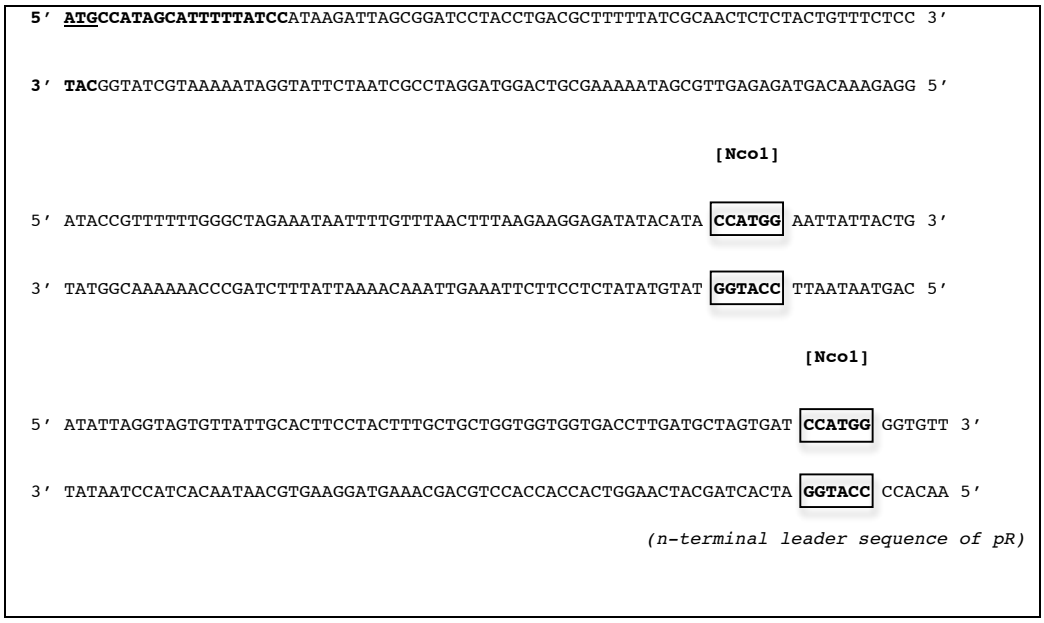
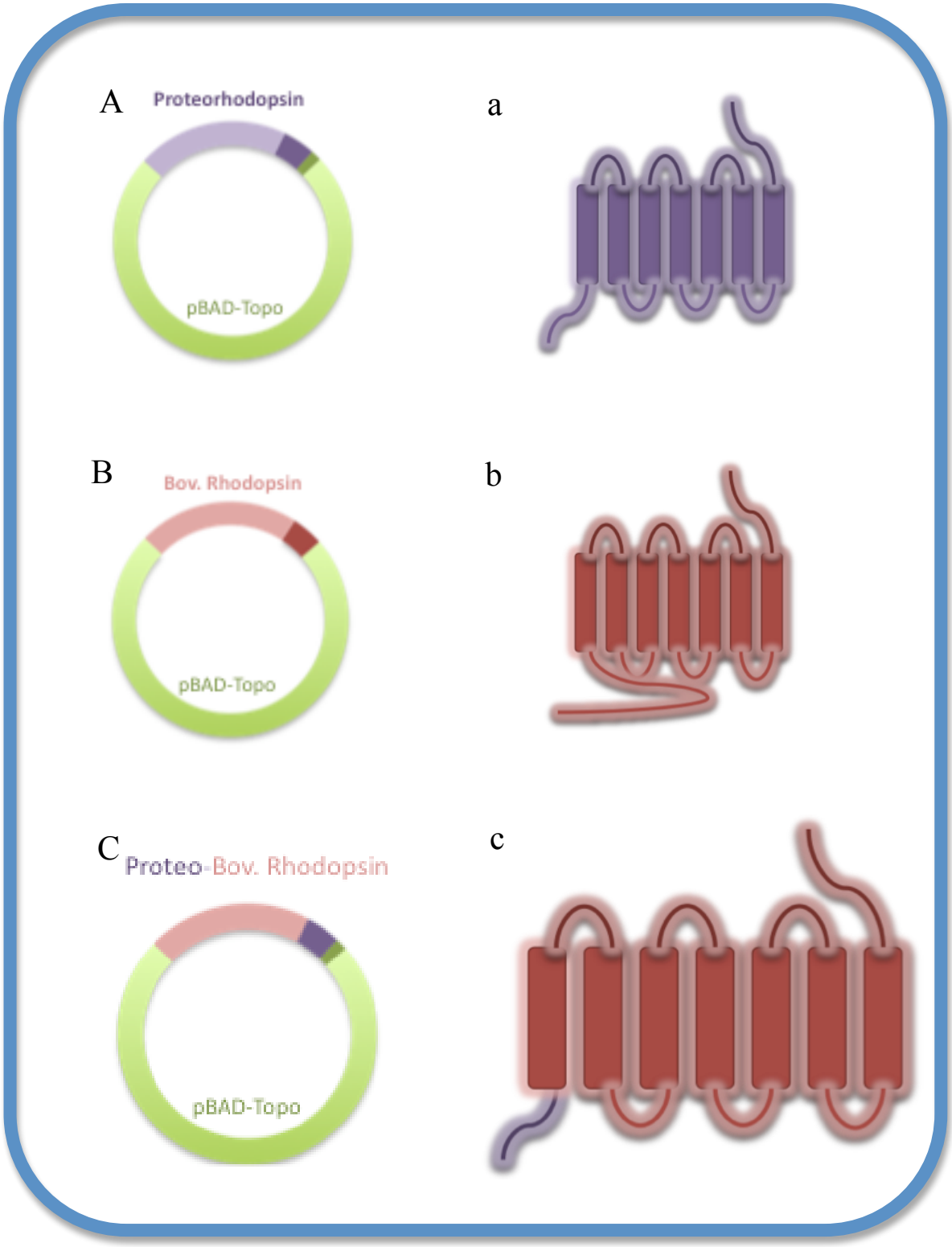


Figure 8 Below shows a schematic diagram of the project. To the left are the plasmids (A,B,C) that encode for the proteins on the right (a,b,c). The chimera, shown on the very bottom ©, is what we are trying to create from the 2 plasmids above it, (A) proteorhodopsin and (B) bovine rhodopsin. We want to successfully express the chimera, on the bottom far right (c) through the experiments mentioned in the paper. Bordering the darker colored region designates the NcoI sites for A, B & C.



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CAPSTONE SUMMARY

Bovine (cow) rhodopsin is a 7-transmembrane (7TM), light-absorbing, protein located in the eye, particularly the rod cells. It is activated by the photoisomerization of retinal, a Vitamin A derivative. This light-triggered reaction activates the G-Protein Coupled Receptor (GPCR), resulting in a signaling cascade within the cell, and usually a physiological response.

When previously cloned in an *E. coli* expression vector in the Braiman Lab, bovine rhodopsin expression was not robust, possibly due to *E. coli*'s lack of recognition of the foreign N-terminus portion of the protein, which may be a prerequisite for proper folding and insertion into the membrane.

Our proposed solution is to create a chimera protein, replacing the N-terminal leader sequence of bovine rhodopsin with proteorhodopsin's N-terminal leader sequence in order to successfully express this chimera in *E. coli* using the pBAD-Topo® plasmid.

Proteorhodopsin (pR), a protein found in marine bacterioplankton, with analogous photochemical activity to bovine rhodopsin, was chosen to serve as a candidate for creating this chimera due to its robust, successful protein expression, giving around 10mg/L of culture, expressed in the same plasmid and bacterial system.

While our focus is on making a protein chimera, we worked with the DNA that encodes for the proteins; therefore, we had to make a

plasmid DNA chimera, and with a restriction enzyme and PCR, the tools were available to do this.

First and foremost, LB media had to be made in order to grow out bacterial cultures, bovine rhodopsin and proteorhodopsin. After incubation of the bacterial cultures, genomic plasmid DNA has to be separately isolated from both cultures.

In order to obtain the N terminus leading sequence of proteorhodopsin, we had to use the polymerase chain reaction (PCR), which exploits the process of DNA replication to yield a large portion of the desired DNA. The primers that were used designated which area we wanted to amplify, which the N terminus leading sequence of proteorhodopsin.

The proteorhodopsin PCR product was then run on a gel to confirm that it worked. It was later digested with NcoI, an endonuclease, for the preparation of creating the plasmid DNA chimera by generating the “sticky ends” needed.

Working with bovine rhodopsin was the next step. The goal was to remove the N terminus leader sequence of this in order that it could be replaced by proteorhodopsin’s. We used NcoI to remove the this part of DNA, which left bovine rhodopsin with two of the same “sticky ends” as proteorhodopsin’s NcoI digested PCR product. This was no coincidence as this served as the pasting site of the vector, bovine rhodopsin, with the insert, proteorhodopsin’s PCR product.

However, the NcoI digested bovine rhodopsin had to undergo an alkaline phosphatase reaction to prevent self re-annealing. Previous attempts of creating a chimera were not successful, so we hypothesized that this would give us a better chance at yielding a product. The way in which the alkaline phosphatase reaction works is opposite of that of a kinase; it removes the phosphate groups through hydrolysis. Following this step, ethanol precipitation was performed to remove unwanted salts.

Finally, the ligation was done on the proteorhodopsin and bovine rhodopsin processed DNA fragments in order to paste the DNA, finalization the creation of the chimera DNA plasmid. After this step was complete, the plasmid was then transformed into bacteria to test if the ligation worked by protein expression, as well as use of the antibiotic, ampicillin.

Topo® plasmid was used to essentially carry bovine rhodopsin. What is useful about this is that it encodes for an antibiotic resistance against ampicillin, which does not permit ampicillin, when treated, to kill off the bacteria. Therefore, if bacterial colonies still proliferate after this antibiotic treatment, it suggests that the plasmid conferring the antibiotic resistance is present, and that the plasmid was successfully transformed into bacteria.

Through gel electrophoresis, I was able to confirm that the DNA from both bacterial cultures, bovine rhodopsin and proteorhodopsin, were isolate. Also, I was able to confirm that the NcoI digest of bovine

rhodopsin and the PCR of proteorhodopsin N terminus leading sequence both succeeded.

Confirming the creation of the chimera is still undergoing in the Braiman Lab. Characterization of protein expression must be done after confirmation of the chimera's creation has been made. Following this, purification of the protein, such as citrate purification, which is being perfect in the Braiman Lab with patent pending, can be used in hopes of creating a pure enough protein to allow its crystallization.